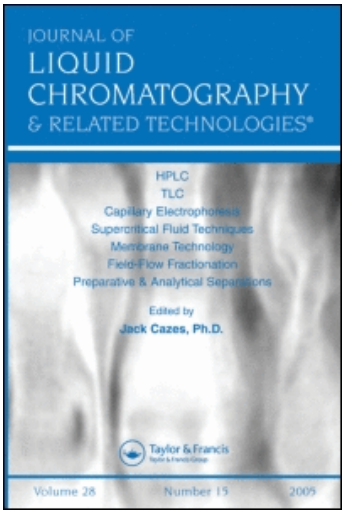

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G.U.E.S.S.—A Generally Useful Estimate of Solvent Systems for CCC

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G.U.E.S.S.—A Generally Useful Estimate of Solvent Systems for CCC

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Abstract: The choice of an appropriate solvent system for Countercurrent Chromatography (CCC) is a critical step in the purification of natural products. Targeted towards their high sample diversity, G.U.E.S.S. is a practical approach for the prediction of CCC distribution constants, K values, by standard thin layer chromatography (TLC). G.U.E.S.S. allows a major reduction in workload by direct use of routine TLC information. The separation capability of CCC focuses on an optimal "window of opportunity" that can be described as the "sweet spot" of CCC separation. The sweet spot of optimal CCC performance may be described as an area where compound K values are between 0.4 and 2.5. Two useful CCC solvent systems: hexane/ethyl acetate/methanol/water and chloroform/methanol/water are organized and recommended as the HEMWat and ChMWat methods of solvent system selection. The relationship of (i) P values, measured by the ratio of UV-vis absorption, (ii) TLC R_f values and (iii) CCC retention volumes for over 20 diverse commercially available natural products are described. The HEMWat method characterizes a versatile solvent selection technique. TLC R_f values will often give practical predictions, even with simple single-phase mixtures. Additional information can be acquired from equivalent solvent systems and by calibration with the G.U.E.S.S. standard compounds. The latter will also aid in the important selection of which phase will function as the mobile phase. The choice of normal *vs.* reverse phase will

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depend on the polarity of compounds that are desired to be gathered into the sweet spot. In addition, G.U.E.S.S. has been shown to be readily applicable to natural product purification necessary for drug discovery, bioassay guided fractionation, and metabolome analysis.

Keywords: Countercurrent chromatography, Bioassay-guided fractionation, Liquid systems metabolomics, Metabolome analysis, Natural products, Drug discovery

INTRODUCTION

CCC Effectiveness

Counter current chromatography (CCC) has emerged as a valuable separation technique in natural products chemistry, as well as other areas. In particular, high speed counter current chromatography (HSCCC) and centrifugal partition chromatography (CPC) have been used to separate and purify a plethora of natural products under diverse conditions in different quantities.[1–21] Despite its indisputable merit, CCC has been passed up for other chromatographic techniques in many laboratories. The major drawback in the employment of CCC separations by both experienced and inexperienced natural products chemists seems to be a paucity of clear guidelines for solvent system selection. The choice of solvent systems for CCC separations is absolutely crucial. Compared to the far more popular solid-support chromatography, the selection of CCC solvent systems is equivalent to choosing both the column and the eluant at once.

The basic requirement for a CCC solvent system is that it consists of two immiscible phases. Many functional solvent systems have been proposed, studied, and successfully employed over the years. One popular method of concocting a solvent system involves the mixing of a hydrocarbon solvent such as hexane with ethyl acetate, methanol, and water. Another very familiar method of arriving at a reasonable solvent system is mixing chloroform, methanol, and water. While CCC does not retain any compounds on the "column," it may not separate many of them in any appreciable way unless the solvent system has been chosen very carefully. There is a "window of opportunity" present in CCC separations that is related to the K value of a given compound in a particular solvent system. The distribution constant, K, can be expressed as the concentration of the compound in the stationary phase divided by the concentration of the compound in the mobile phase. A solvent system, where the K value of a particular compound is close to 1, is considered to be the ideal system for separating the compound. According to Figure 1, small K values result in a loss of peak resolution, while large K values tend to produce excessive sample band broadening and long run times.[22] In addition, the decision of which phase (upper or lower) will be the mobile phase is less important if $K = 1$, since the retention

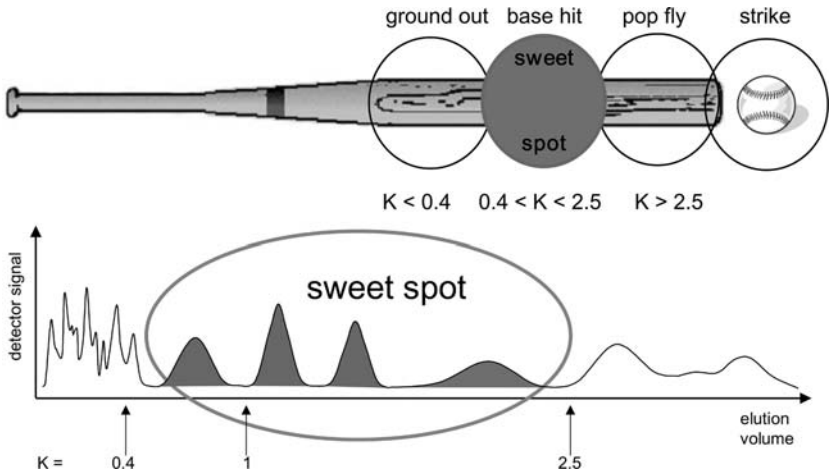

Figure 1. Schematic of the CCC sweet spot. The range around K = 1 provides optimum resolution and, in analogy to bat and racket sports, can be called the sweet spot of separation. Therefore, the process of choosing a solvent system aims to find a mixture, in which the analyte elutes in the range between K = 0.4 and K = 2.5. The latter is a working definition of the sweet spot limits for the purpose of this study.

volume of the target compound will be very similar in either mode. The window of opportunity presented by CCC separations may be compared to the "sweet spot" of bat and racket sports (Figure 1). The sweet spot is the area of the racket or bat that offers the optimum return for effort invested. Missing the sweet spot may result in missing the ball altogether. Hitting the ball outside of the sweet spot may "get the job done" in some cases, but not with the elegance and power of hitting it in the sweet spot. A working definition of the sweet spot in CCC is the interval of K values between 0.4 and 2.5.

Solvent System Selection Methodology

A reliable method of solvent selection should be available that is accessible by both experienced CCC users and neophytes. An ideal method of selecting an appropriate solvent system for a CCC separation would satisfactorily address the following criteria: (i) systematic in its approach, (ii) versatile for a wide range of natural products, (iii) supple enough to allow some "wiggle room" in making a judgment, (iv) time efficient, (v) adaptable to rational fine-tuning, (vi) applicable to mixtures of unknown composition, as well as samples of known composition.

Since thin layer chromatography (TLC) has traditionally played the role as solvent system selection method in solid-support chromatography, a method that involves the estimation of CCC solvent system choice, based on TLC

behavior, may meet the above criteria with some degree of satisfaction. Therefore, the goal of this study was to implement a TLC-based method for the generally useful estimation of solvent systems in CCC, allowing a good first "G.U.E.S.S.," and be able to replace conventional procedures (see Figure 2). Without a doubt, TLC is a common denominator of all natural products separations. Samples ranging from crude extracts to purified compounds are subjected to TLC as a quick and easy way to assess their composition, identity, and purity. Many useful TLC solvent systems are known and routinely used in laboratories all over the world. In fact, the G.U.E.S.S. method has been done in reverse for decades. It is customary to separate an extract or column fraction by CCC, and then perform TLC on the collected CCC fractions in order to ascertain their composition and purity as seen in Figure 2. If TLC can be routinely used to analyze CCC fractions, then it should be possible to use TLC to predict CCC elution performance. However, relating TLC and CCC is fundamentally challenging, since their respective physicochemical means of separating compounds is quite different. At least one method of predicting droplet counter current chromato-

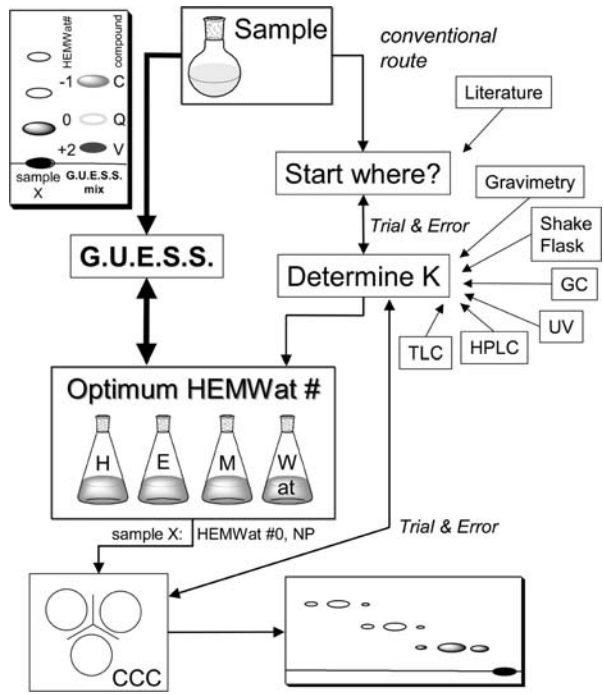

Figure 2. Schematic representation of the CCC protocol with the G.U.E.S.S. method in comparison to the conventional approach of determining the optimum CCC solvent system (HEMWat in this example).

graphy (DCCC) behavior based on TLC observations has been proposed.[23] In this method, silica gel TLC was done with the organic layer of a chloroform/methanol/water biphasic solvent system in order to predict the best mobile phase for optimal DCCC performance in that solvent system.

Over the years, several other methods of solvent system selection for CCC have been proposed, studied, and utilized. An accepted method of predicting CCC behavior is to perform a partitioning study of a compound by measuring the relative concentrations of the compound in the upper and lower layers of a biphasic solvent system. The partition coefficient, P, can be expressed as the concentration of the compound in the upper phase divided by the concentration of the compound in the lower phase. P values obtained by partitioning studies predict the retention time of a particular compound, e.g., in an HSCCC instrument, when the proper consideration is made for the mobile and stationary phase of the HSCCC run.

The most common form of partition study is descriptively called the "shake-flask" method. This method involves dissolving a small amount of a compound or mixture in a biphasic system, shaking them together, and allowing the system to equilibrate before measuring the concentration of the target compound(s) in each layer. The concentration in each layer can be measured by three principle methods (see Figure 2): (i) The two phases may be separated and the solvents evaporated in order to obtain the mass of the residues. This gravimetric method requires relatively large amounts of compound to get a reliable result. It is also not very useful for mixtures, which may contain large amounts of extraneous compounds. (ii) The relative concentrations can be measured by measuring the UV-vis absorption of each layer. This spectroscopic method works well for targeting a particular chromophore by itself, or in a mixture of non-absorbing compounds. It can be done with small amounts of compounds. However, the spectroscopic method does not work for compounds that do not absorb in UV-vis and for mixtures where compounds' absorptions interfere with each other. Also, since the compound is being measured in two different solvents, steps must be taken to minimize solvent interference with spectroscopic measurements. (iii) In the case of mixtures, each phase can be analyzed by high pressure liquid chromatography (HPLC) or gas chromatography (GC), and the relative amounts of the compounds present in each layer can be determined. This chromatographic method requires the development of a reliable HPLC or GC protocol that gives a reasonable separation of the compounds of interest. The chromatographic method is relatively time consuming when several solvent systems must be tried. In addition, for many natural product samples the target analyte may not even be known, such as is always the case in bioassay-guided fractionation.

No matter how efficient or reliable the shake-flask method may be, the problem of "where to start" still needs to be addressed (see Figure 2). The same bewildering choice of solvent systems is present when choosing the solvent system for a shake-flask partition study as it is for

a CCC separation. Therefore, the TLC based G.U.E.S.S. system is at least complementary to the shake-flask method, and at best can replace the shake-flask and similar methods. Another advantage is that, considering the complexity of the detection methods outlined above, CCC so far is dependent on the performance of other high-resolution chromatographic techniques instead of standing on its own feet. All together, this substantiates the demand for a simple, more self-sufficient approach such as the G.U.E.S.S. method.

EXPERIMENTAL

Apparatus

The UV-vis spectrophotometry for partitioning studies was performed with a dual beam Beckman DU 7400 scanning spectrophotometer. High speed countercurrent chromatography was carried out using a J-type instrument (Model CCC-1000; Pharma-Tech Research Corporation, Baltimore, MD, USA) containing a self-balancing three-coil centrifuge rotor equipped with 3×108 or 3×283 mL columns, the internal diameters of PTFE teflon tubing were 1.6 mm and 2.6 mm, respectively. The revolution radius of the distance between the holder axis and central axis of the centrifuge (R) was 7.5 cm, and the β-value varied from 0.47 at the internal terminal to 0.73 at the external terminal ($\beta = r/R$ where r is the distance from the coil to the holder shaft). The HSCCC system was equipped with a Lab-Alliance Series III digital single-piston solvent pump, a Shimadzu SPD-10A UV-vis detector with preparative flow cell, a Cole-Parmer modular paperless recorder model 80807-00, and a Pharmacia Biotech RediFrac 95-tube fraction collector.

Analytical TLC was performed at room temperature on Alugram precoated 0.20 mm thick silica gel G/UV_{254} aluminum plates (20×20 cm; Macherey-Nagel, Germany). Plates were cut to 9.5 cm length and various widths before spotting. TLC experiments were carried out in duplicate. Plates were dipped in general-purpose reagent *p*-anisaldehyde/sulfuric acid/acetic acid 1/1/48, drained and heated on a Camag TLC Plate Heater III at 95°C for about 5 minutes. All TLC chromatograms were scanned at 150 dpi with a Canon CanoScan N670U scanner.

Solvents and Reagents

All solvents were HPLC grade from Fisher Scientific or Sigma-Aldrich. Chemicals used, including the commercially available G.U.E.S.S. reference standards, were purchased from the Sigma Aldrich Fluka group (St. Louis, MO, and Milwaukee, WI).

Volume Ratios and Settling Times

Volume ratios were determined by adding appropriate volumes of hexane, ethyl acetate, methanol and water to a 125 mL separatory funnel to equal a (premixed) combined volume of 100 mL. Each solvent system was thoroughly equilibrated at room temperature by repeated shaking and degassing. The solvent mixture was transferred to a 100 mL graduated cylinder to measure the volume ratio of the two phases. The experiment was repeated 3 times to obtain the mean value. Settling times were determined by adding appropriate volumes of hexane, ethyl acetate, methanol and water to a 5 mL test tube to equal a (premixed) combined volume of 4 mL. The test tube was stoppered and the solvent was gently mixed by inverting the test tube 5 times. After mixing, the test tube was immediately placed in a vertical position, and the time required for the solvent mixture to settle into two clear layers was measured. The experiment was repeated 5 times to obtain the mean value.

Partitioning Studies

Approximately 1 mg of compound was added to a 12 × 75 mm test tube. Appropriate volumes of various solvents were added with a Pipet-Lite™ pipette (Rainin Instrument, LLC) to make a combined volume of 2 mL. The test tube was stoppered and shaken two minutes with a vortex mixer. Test tubes were routinely centrifuged for about one minute to break any emulsions present. The UV-vis analysis was performed by removing 50 microL from the bottom phase and mixing it with 2 mL of methanol in a spectrophotometer cuvette. The sample was scanned from 210 to 400 nm against an appropriate blank. The same procedure was repeated for the upper phase. Two test tubes were prepared for each solvent system and two UV-vis trials were done with each phase from each test tube. P values were calculated by dividing the absorbance measured for the upper phase by the absorbance measured for the bottom layer at the same wavelength (lambda max) for each. Results were averaged for determination of the final P value.

Terminology

The distribution constant, K, is calculated by taking the difference of the retention volume of a particular compound and the mobile phase volume and dividing by the volume of the stationary phase. K may also be expressed as the concentration of the compound in the stationary phase divided by the concentration of the compound in the mobile phase. Accordingly, the K value is dependant on which phase (upper or lower) is chosen as the mobile phase for a particular CCC separation (normal phase vs. reverse phase, see below). In contrast, the P value is independent of the

mobile phase selection, and is expressed as the concentration of the compound in the upper phase divided by the concentration of the compound in the lower phase of a stationary binary system. In order to relate shake-flask P values to TLC R_f values (the R_f value is the distance between a spot and the origin divided by the distance between the solvent front and the origin), P is expressed in terms of P_f equal to the concentration of the compound in organic phase divided by the sum of concentrations of the compound in both phases of a binary system. Consequently, P and K, P_f and R_f, as well as LogP may be used to describe the sweet spot as given in Table 1.

HSCCC of G.U.E.S.S. Standard Compounds

A mixture of compounds was prepared with 1–5 mg of each compound added to 4 mL of the biphasic solvent system. The mixture was filtered and loaded into a 5 mL sample loop. All solvent systems were thoroughly mixed, vented and allowed to separate into two distinct phases before use. The HSCCC tubing (320 mL) was first filled with the stationary phase. The coils were rotated 800 rpm as the mobile phase was pumped at a flow rate of 1.5 mL/min. In order to observe the percent retention of stationary phase in the column, the resulting effluent was collected in a graduated cylinder. When the volumes of the two phases of the eluant were approximately equal, the hydrodynamic equilibrium was understood to be established. To begin the run, the standard compound mixture was injected on the column. A UV-vis detector monitored the eluant, and all fractions were collected at 3 min/tube. The collected fractions were reduced in volume and TLC performed to corroborate the UV-vis data.

Separation of *Valeriana officinalis* Analytes

Powdered methanolic extract of *Valeriana officinalis* roots was separated on silica gel (MN Kieslgel 60) vacuum column (10 × 30 cm) using a gradient of hexane, ethyl acetate, methanol and water beginning with 100% hexane and ending with methanol/water (6/4). Column fractions were reduced in

Table 1. Summary of sweet spot parameters used in the definition of G.U.E.S.S.

	Lower limit of sweet spot	Optimal value	Upper limit of sweet spot
P or K	0.4	1	2.5
Log P	−0.4	0	0.4
P_f or R_f	0.29	0.5	0.71

volume and monitored by TLC. Combined fractions corresponding to hexane/ethyl acetate 2/1 and 2/3 were separated by HSCCC to isolate valerenic acid and acetoxy valerenic acid, respectively. HSCCC separations of *V. officinalis* analytes was carried out as described above in normal phase (tail in head out) mode with a 25 mL sample loop, total coil capacity of 850 mL, and 3 mL/min flow rate.

RESULTS AND DISCUSSION

This study was divided into 4 stages: (i) The behavior of commercially available natural products (G.U.E.S.S. standard compounds) in hexane/ethyl acetate/methanol/water solvent systems was examined by the shake-flask method. (ii) The relationship between TLC R_f values and shake flask P values of the G.U.E.S.S. standard compounds was correlated, and a methodology for using G.U.E.S.S. to determine the best solvent system for CCC separation was developed. (iii) The behavior of the standard G.U.E.S.S. compounds in HSCCC was observed in order to investigate the efficacy of the G.U.E.S.S. method, and further explore the necessary parameters to consider when selecting a solvent system for an optimal CCC separation. (iv) The G.U.E.S.S. method was employed to determine the best solvent systems for HSCCC separations of natural products from valerian extracts.

The HEMWat Method in G.U.E.S.S.

Hexane/ethyl acetate/methanol/water solvent systems have widely been used to separate a variety natural products such as theaflavins, catechins, flavonoids, polyphenols, diterpenes, flavonoid glycosides, ivermectins and macrolide antibiotics.[1–14] The proposed HEMWat method based on a progression of hexane/ethyl acetate/methanol/water solvent systems was inspired by previously published solvent system arrays.[24] The HEMWat method was designed to provide a systematic process of choosing a CCC solvent system for separating a wide range of organic compounds of low and medium polarity. In the proposed method the volume of hexane and ethyl acetate is constant and equal to the volume of methanol and water. The polarity of the system increases as the numbers (-7 to $+8$) designated for each solvent system become more positive (Table 2). In the HEMWat method the organic phase is mainly composed of hexane and ethyl acetate in the upper phase of the biphasic mixture, while the aqueous phase is mainly composed of methanol and water in the lower phase of biphasic mixture (Table 1).[25]

An established way of determining the fitness of solvent systems is to determine the volume ratios and settling time.[24] Table 2 describes the

Table 2. HEMWat volume ratios and settling times

HEMWat system #	Hexane (H)	EtOAc (E)	Methanol (M)	Water (Wat)	Volume ratio (U/L)	Settling time (seconds)
−7	9	1	9	1	0.72	11
−6	8	2	8	2	0.73	13
−5	7	3	7	3	0.69	13
−4	7	3	6	4	0.76	10
−3	6	4	6	4	0.68	14
−2	7	3	5	5	0.83	18
−1	6	4	5	5	0.76	22
0	5	5	5	5	0.71	27
+1	4	6	5	5	0.68	21
+2	3	7	5	5	0.67	28
+3	4	6	4	6	0.83	20
+4	3	7	4	6	0.83	18
+5	3	7	3	7	0.91	30
+6	2	8	2	8	0.93	33
+7	1	9	1	9	0.91	15
+8	0	10	0	10	0.95	10

solvent composition, volume ratios and settling times for HEMWat method solvent systems. For practical purposes, the volume ratio (upper phase volume divided by lower phase volume) of a CCC biphasic solvent system should be as close to 1 as possible. This means that nearly equal amounts of upper phase and lower phase would be available for use in mobile and stationary phases as needed. A rapid settling time of about 30 seconds or less would allow the phases to mix and separate suitably under the conditions presented by the CCC instrument. The settling time is simply measured by observing the time required for the two phases to completely separate in a shaken test tube. HSCCC stationary phase retention data, for HEMWat −6, −3, 0, +3, +6 and +7 at different flow rates, has been previously published.[26]

The first stage of this study was accomplished by measuring the P value (as defined in the experimental section) for 22 natural products that comprise the G.U.E.S.S. Mix reference standards, (see Figure 3) over the complete range of HEMWat solvent systems. The simple "shake-flask" process was used to distribute the commercially available compounds between the two phases of a HEMWat solvent system. All of the compounds tested showed similar trends in HEMWat systems. Generally, as the lower phase becomes more aqueous (the HEMWat number becomes more positive and the sweet spot becomes more polar), organic compounds tend to flee towards the upper phase and P increases. An exponential increase in P as the HEMWat system becomes more positive can be observed by graphing the $\log_{10}$ of P

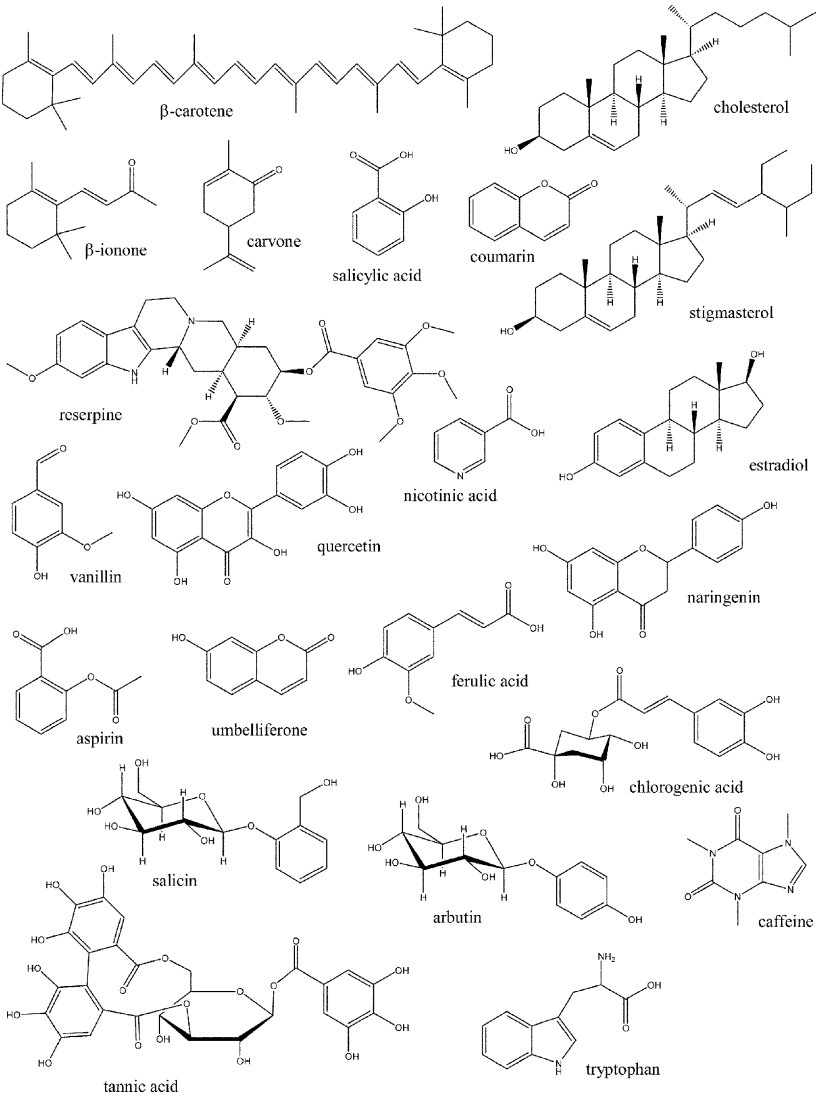

Figure 3. The G.U.E.S.S. Mix reference standards used in this study.

versus the HEMWat number. Figure 4 presents umbelliferone LogP values in HEMWat systems as determined by the shake-flask method.

The linearity of the resultant LogP plots, such as the one for umbelliferone shown in Figure 4, is remarkable, considering that it is not obvious that the HEMWat solvent systems increase by regular intervals of polarity. Of course, the P value is not strictly a measure of relative polarity, but rather the result of a

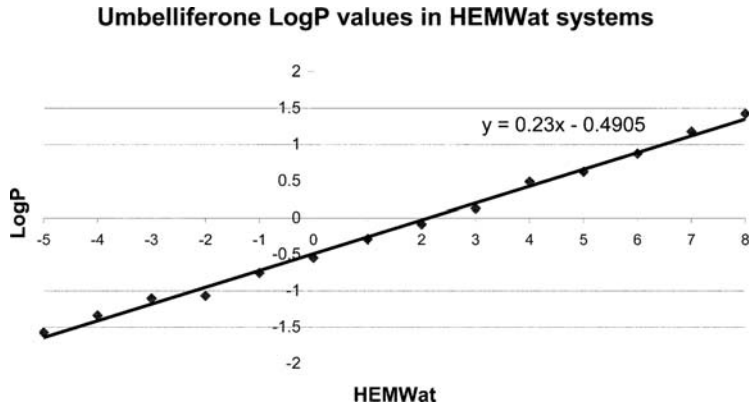

Figure 4. Umbelliferone LogP values in HEMWat systems as determined by the shake-flask method.

complex interaction of a particular compound's relative solubilities in 4 different solvents. Interestingly, as can be seen in Figure 5, most of the compounds tested showed nearly linear Log P behavior with similar slopes.

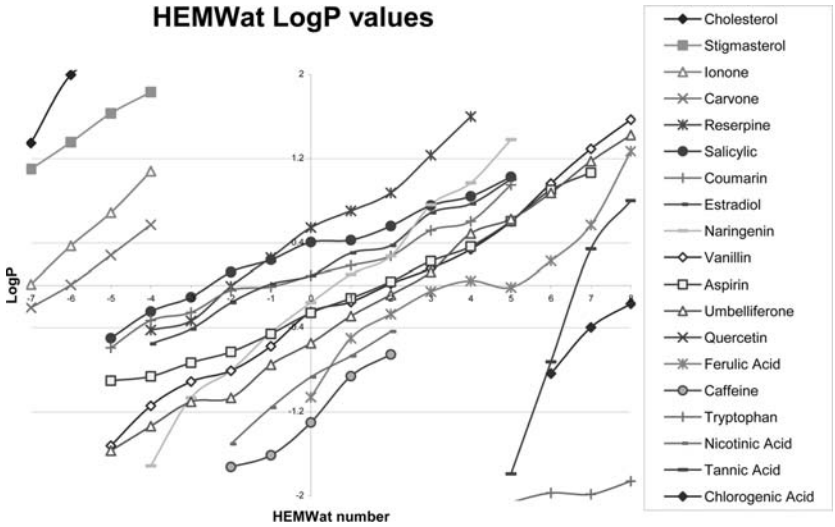

Figure 5. Partition coefficients of 19 of the natural products used in the G.U.E.S.S. method in HEMWat solvent systems as determined by the shake-flask method. Used in a mixture (G.U.E.S.S. Mix) the compounds may be employed as TLC calibration standards to determine the best HEMWat solvent system for CCC separations.

Table 3. Ideal HEMWat number for 13 G.U.E.S.S. standard compounds as determined by the shake-flask method

Code	Compound	HEMWat # with P = 1
I	β-Ionone	−7
O	Carvone	−6
Z	Salicylic acid	−3
R	Reserpine	−2
M	Coumarin	−1
E	Estradiol	−1
Q	Quercetin	0
N	Naringenin	+1
V	Vanillin	+2
A	Aspirin	+2
U	Umbelliferone	+2
F	Ferulic acid	+4
J	Tannic acid	+7

The proposed HEMWat series of CCC solvent systems demonstrated its versatility by showing that many compounds, such as those shown in Table 3, have a P value equal to 1 in the range of HEMWat solvent systems. This means that many natural products are likely to be satisfactorily separated in one HEMWat solvent system or another. In addition, some compounds have LogP values within the sweet spot ($-0.4 < \text{LogP} < 0.4$ which is the same as $0.4 < P < 2.5$) in one or more HEMWat solvent systems, even though they do not have an ideal $P = 1$ value in any HEMWat solvent system. The currently proposed HEMWat method is a versatile and useful method for the separation of a variety of natural products, with polarities ranging from medium lipophilic to slightly polar (non-glycosidic).

The ChMWat Method in G.U.E.S.S.

Chloroform/methanol/water systems have typically been used to separate more polar natural products such as alkaloids, phenolics, phenolic glucosides, flavonoids, flavonoid glucosides, anthraglycosides, and anthraquinones.[15–21] The ChMWat method, designed to provide a systematic process of choosing a CCC solvent system for the separation a wide range of organic compounds of medium and high polarity, has been previously described.[24] In this method (see Table 4) the volume of chloroform stays constant and equal to the sum of methanol and water volumes. The proportion of methanol to water increases incrementally from 0/10 to 7/3.

Table 4. ChMWat system numbering and solvent ratios

ChMWat system	$CHCl_3$	MeOH	Water
−3	10	0	10
−2	10	1	9
−1	10	2	8
0	10	3	7
+1	10	4	6
+2	10	5	5
+3	10	6	4
+4	10	7	3

Unlike the HEMWat system, four different trends are observed as P values for a single compound are compared in successive ChMWat solvent systems:

1. Exponential increase comparable to HEMWat: As the amount of methanol in the upper aqueous phase increases, (ChMWat system becomes more positive) organic compounds tend to become more attracted to the methanol/water upper phase and P increases. These compounds, exhibited in Figure 6, represent the most hydrophobic compounds that have measurable P values with the shake-flask method.

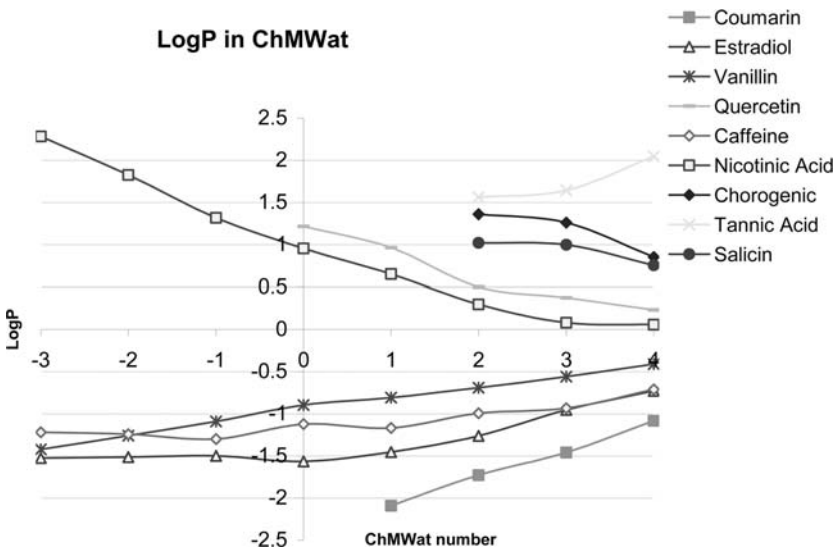

Figure 6. Partition coefficients for 9 G.U.E.S.S. standard compounds that fall in the polarity range of the ChMWat solvent systems as determined by the shake-flask method.

2. Exponential decrease different from HEMWat: As the amount of methanol in the upper aqueous phase increases (ChMWat system becomes more positive), some compounds tend to become more attracted to the chloroform lower phase and P decreases (Figure 6). This trend has been previously demonstrated with indole-3-acetamide (IA) and indole-3-carboxylic acid (ICA).[24]
3. Concave correlation: Initially, relative solubility in the upper aqueous layer decreases as the proportion of methanol increases. As the relative volumes of methanol and water become equal, the trend reverses and the compound becomes more soluble in the upper aqueous layer as the proportion of methanol increases. This trend, illustrated in Figure 7, has previously been observed with various DNP derivatized amino acids.[24]
4. Convex correlation: Initially, the relative solubility of tryptophan in the upper aqueous layer increases as the proportion of methanol increases. As the relative volumes of methanol and water become equal, the trend reverses and tryptophan becomes less soluble in the upper aqueous layer as the proportion of methanol increases (Figure 7).

Versatility of the ChMWat Method

No compounds in this study had $P = 1$ in any ChMWat system. However, the seven compounds in Table 5 had P values in the range of 0.4 to 2.5 in ChMWat +4.

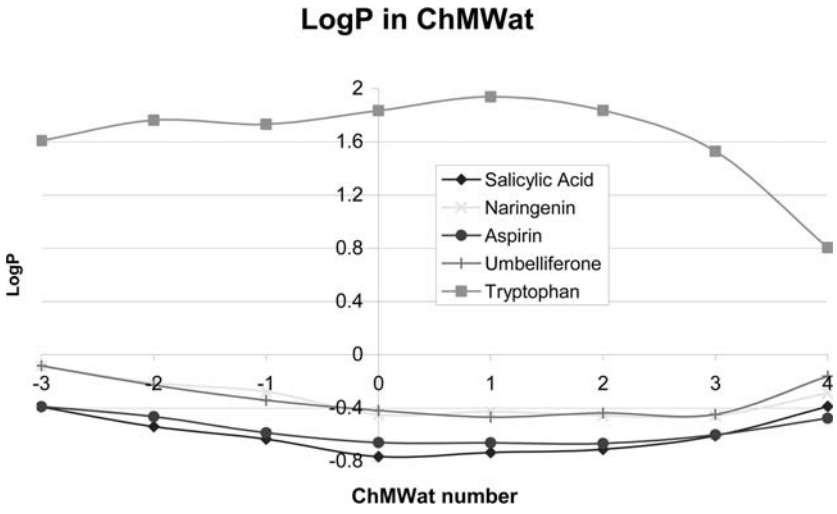

Figure 7. Partition coefficients for salicylic acid, naringenin, aspirin, umbelliferone and tryptophan in ChMWat solvent systems as determined by the shake-flask method.

Table 5. Partition coefficients in of salicylic acid, naringenin, vanillin, umbelliferone, quercetin, ferulic acid and nicotinic acid in ChMWat +4

Code	Compound	P in ChMWat +4
S	Salicylic acid	0.4
V	Vanillin	0.4
N	Naringenin	0.5
U	Umbelliferone	0.7
D	Nicotinic acid	1.1
F	Ferulic acid	1.5
Q	Quercetin	1.7

The second stage of this study involved establishing a link between shake-flask partition coefficients, P, and TLC R_f values. An obvious approach to establishing this link is to compare the shake-flask P value of a compound in a particular HEMWat solvent system with the TLC R_f value of that compound developed in the organic phase of the HEMWat solvent system.[23] In order to simplify the TLC, the HEMWat organic phase was replaced with a solvent system made by simply mixing hexane and ethyl acetate in the same ratio as the HEMWat solvent system. Table 6 describes 16 HEMWat systems that correspond to 10 SSE (solvent systems based on ethyl acetate) systems. For example, HEMWat −5, −4, and −2 all correspond to a hexane/ethyl acetate ratio of 7/3 (Table 6).

Table 6. Equivalence of HEMWat and SSE solvent systems

HEMWat	nHex	EtOAc	MeOH	Water	SSE	nHex	EtOAc
−7	9	1	9	1	1	9	1
−6	8	2	8	2	2	8	2
−5	7	3	7	3	3	7	3
−4	7	3	6	4	3	7	3
−3	6	4	6	4	4	6	4
−2	7	3	5	5	3	7	3
−1	6	4	5	5	4	6	4
0	5	5	5	5	5	5	5
+1	4	6	5	5	6	4	6
+2	3	7	5	5	7	3	7
+3	4	6	4	6	6	4	6
+4	3	7	4	6	7	3	7
+5	3	7	3	7	7	3	7
+6	2	8	2	8	8	2	8
+7	1	9	1	9	9	1	9
+8	0	10	0	10	10	0	10

Most of the compounds showed a rather close correlation between HEMWat TLC R_f, SSE TLC R_f, and HEMWat P_f values. For example, in the case of estradiol shown in Figure 8, the SSE $R_f = 0.5$ (ideal R_f value) falls between SSE 4 and SSE 5, suggesting possible ideal HEMWat numbers of -3, -1 or 0. Given that information, the best G.U.E.S.S. would be HEMWat -1 as the optimal solvent system for a separation involving estradiol. The HEMWat TLC results further validate the SSE TLC results by suggesting an ideal HEMWat number of 0. The G.U.E.S.S. method allows for some "wiggle room" in selecting a solvent system. In this case, either HEMWat -1 or 0 would be appropriate solvent systems to try. In fact, the HEMWat shake-flask P value plot implies that, in the case of estradiol, the sweet spot of $0.29 < P_f < 0.76$ ($0.4 < P < 2.5$) is between HEMWat numbers -2 and $+2$. Therefore, any solvent system in that range is likely to give a reasonable separation of estradiol (Figure 8).

Interestingly, compounds tend to have higher R_f values in TLC with a simple hexane/ethyl acetate mixture than in the corresponding HEMWat organic phase with the same hexane/ethyl acetate ratio. The HEMWat organic upper phase certainly contains a small amount of methanol and water, so it may be more polar than the simple hexane/ethyl acetate mix. However, the volume ratios of upper/lower shown in Table 2 suggest that the organic phase loses a significant amount of ethyl acetate relative to hexane to the aqueous phase. We would not expect that simplifying the TLC solvent system from the HEMWat organic phase to the SSE series would improve the ideal HEMWat number prediction, but it certainly still tends to arrive at a number in the partitioning sweet spot. It does not appear to be helpful to shift or reformulate the SSE solvent systems relative to the HEMWat solvent systems in order to give them a better fit. As the example of estradiol shows, some correlations are already quite good between SSE TLC R_f and shake-flask P_f values. In fact, for all 13 G.U.E.S.S. standard compounds in Table 3, the G.U.E.S.S. method will successfully predict an ideal HEMWat number in the shake-flask P value sweet spot. It is interesting

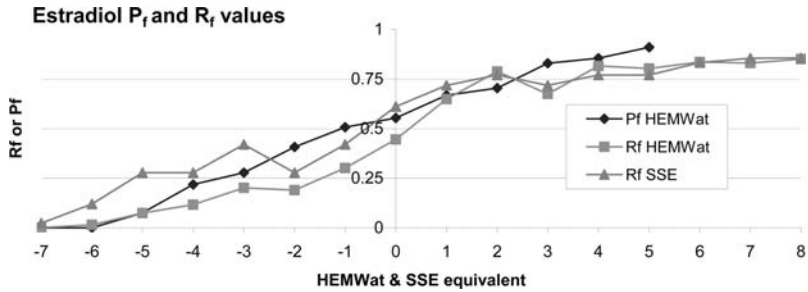

Figure 8. Comparison of estradiol P_f and R_f values in HEMWat and SSE.

to note that the shape of curves tends to be different between R_f and P_f values, possibly indicating a different manner of interaction between mobile and stationary phases of TLC and the two liquid phases of liquid-liquid partitioning (different selectivity).

The G.U.E.S.S. HEMWat method can also predict what compounds will not be well separated in any HEMWat solvent system. For instance, carotene is clearly too lipophilic to be well separated by the HEMWat method. Examples of compounds that are too hydrophilic to be well separated by HEMWat systems are caffeine, nicotinic acid, tryptophan, salicin, chlorogenic acid and arbutin. As a note of caution, this method renders misjudgments for cholesterol, stigmasterol, reserpine, and tannic acid, compounds that behave like more lipophilic compounds in shake-flask partitioning than the TLC suggests. Even so, the trends between TLC and partitioning in the HEMWat solvent systems are surprisingly similar. There is a fair agreement between the R_f and P_f values over the breadth of HEMWat solvent systems.

Using TLC Standards to Calibrate the G.U.E.S.S. Method

In addition to the aforementioned correlation, it is useful to calibrate the HEMWat method by a direct comparison of an unknown compound or mixture to a cocktail of G.U.E.S.S. standard compounds on the TLC chromatogram (Figure 2). In this way, some additional clues as to the CCC tendencies may arise that are obscured by simply finding a SSE solvent system with a P_f value near 0.5. Another advantage of using standard compounds is that, practically speaking, TLC R_f values tend to vary depending on TLC plate quality, solvent quality and diverse developing conditions. The major advantage of using the G.U.E.S.S. standards for the prediction of suitable HEMWat systems is that they can be applied in any TLC screening of the sample, which may be the fraction control of a preceding chromatographic separation, or a simple TLC screening of crude materials. All that is required is the co-spotting of the appropriate G.U.E.S.S. Mix standards. Standard compounds allow expansion of the range of solvent systems that may be used. For example, Table 7 shows chloroform/methanol/water (SSC) and toluene/acetone (SST) systems that may be considered to be equivalent to hexane/ethyl acetate (SSE). Figure 9 shows that the R_f values for coumarin are nearly the same for three equivalent solvent systems proposed in Table 7 (see Fig. 9).

Equivalent solvent systems may be used to predict CCC behavior in the case of nitrogen containing compounds such as reserpine, caffeine and nicotinic acid. Alkaloids, such as those shown in Table 8, tend to give SSE TLC R_f values that are much lower than the TLC R_f values in the equivalent chloroform/methanol/water (SSC) solvent systems when compared with non-alkaloids such as vanillin and umbelliferone.

Table 7. SSE-G.U.E.S.S. with equivalent TLC solvent systems

Hexane/ ethyl acetate	Chloroform/methanol/ water	Toluene/acetone
SSE1 90/10	SSC1 100/0/0	
SSE2 80/20	SSC2 99/1/0	
SSE3 70/30	SSC3 98/2/0	SST3 90/10
SSE4 60/40	SSC4 95/5/0	SST4 80/20
SSE5 50/50	SSC5 90/10/0.5	SST5 70/30
SSE6 40/60	SSC6 85/15/0.5	SST6 60/40
SSE7 30/70	SSC7 80/19/1	SST7 50/50
SSE8 20/80	SSC8 75/24/1	
SSE9 10/90	SSC9 60/39/1	

G.U.E.S.S. Correlates ChMWat P Values with TLC R_f Values

Equivalent chloroform/methanol/water (SSC) solvent systems shown in Table 9, are comparable to the corresponding ChMWat organic phase in TLC development.

However, ChMWat solvent systems show much wider discrepancies than HEMWat solvent systems between shake-flask P_f and TLC R_f values. Partition coefficients for most of the compounds tested were high in ChMWat solvent systems, because most lipophilic to slightly polar organic molecules are quite soluble in chloroform. On the other hand, chloroform is a non-polar TLC solvent, which needs the inclusion of methanol and/or water to transport most organic compounds. Naringenin, as shown in Figure 10, is an example of this discrepancy, because it exhibits a much greater affinity for the chloroform phase than the TLC results predict. Most of the compounds tested exhibit similar behavior.

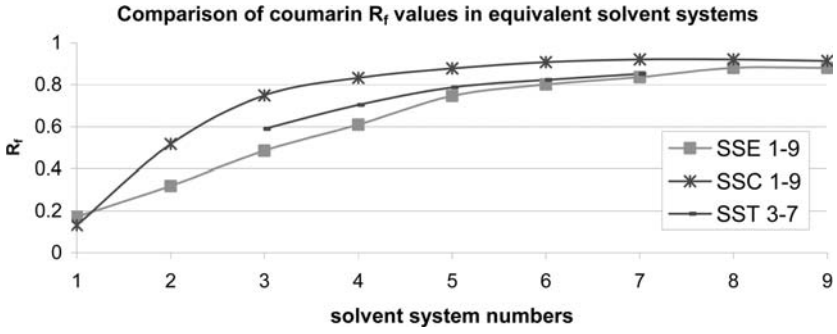

Figure 9. Comparison of coumarin R_f values in equivalent solvent systems.

Table 8. Comparison of R_f values in equivalent solvent systems for reserpine, caffeine, nicotinic acid, umbelliferone and vanillin

Code	Compound	SSE5 R_f value	SSC5 R_f value
R	Reserpine	0.08	0.65
C	Caffeine	0.04	0.65
D	Nicotinic acid	0.12	0.27
U	Umbelliferone	0.57	0.58
V	Vanillin	0.68	0.77

Using TLC of ChMWat organic phase or a simple chloroform/methanol/water mix to approximate the organic layer composition, is apparently less effective than the HEMWat method in predicting CCC behavior for many compounds. However, it may be useful for some types of compounds and when comparing analytes of the same compound class. For example, ChMWat systems nicotinic acid, shown in Figure 11, exhibits a reasonable correlation between shake-flask P_f and TLC R_f values.

Normal and Reverse Phase Elution

The third stage of this study, after the shake-flask partitioning and TLC experiments, was the observation of the HSCCC behavior of the standard compounds. This was done in order to further explore the necessary parameters to consider when selecting a solvent system for an optimal CCC separation. The most straightforward way to express the CCC behavior of compounds is that the more soluble a compound is in the mobile phase, the more quickly it will elute. In HEMWat systems with the organic phase as the mobile phase, generally, the less polar compounds elute faster than the more polar compounds. This is comparable to the "normal" phase of solid

Table 9. ChMWat and SCC equivalent solvent systems

ChMWat	$CHCl_3$	CH_3OH	H_2O	SSC	$CHCl_3$	CH_3OH	H_2O
−3	100	0	0	1	100	0	0
−2	100	10	90	2	99	1	0
−1	100	20	80	3	98	2	0
0	100	30	70	4	95	5	0
+1	100	40	60	5	90	10	0.5
+2	100	50	50	6	85	15	0.5
+3	100	60	40	7	80	19	1
+4	100	70	30	8	75	24	1

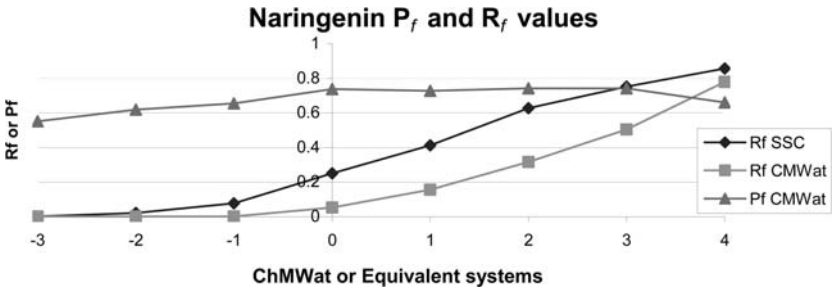

Figure 10. Comparison of naringenin P_f and R_f values in ChMWat and SSC solvent systems.

support liquid chromatography. The counter current chromatography equations that relate the retention volume of a particular compound to the mobile phase and stationary phase volume of a particular set of HSCCC conditions can be written as follows:

$$V_{ret} = V_{mob} + K \cdot V_{stat} \Leftrightarrow K = \frac{V_{ret} - V_{mob}}{V_{stat}}$$

The relationship between the shake-flask partition coefficient $P = c_{UP}/c_{LP}$ and $K = c_{stat}/c_{mob}$ is $K = 1/P$, since the upper phase is mobile and the lower phase is stationary, HEMWat systems.

An observation can be made with reference to reasonable retention times and solvent usage. With a 320 mL coil and 1.5 mL/minute flow rate, one column volume is eluted in 3 hours and 33 minutes. With an 80% stationary phase retention, a compound with $K = 2$ would take almost two column volumes (576 mL) to elute, or 6 hours and 24 minutes. Experience shows that this is liable to be the upper limit for a run time at this flow rate, since peaks tend to get more spread out as retention time increases, and large amounts of solvents are necessary. Another practical observation is that

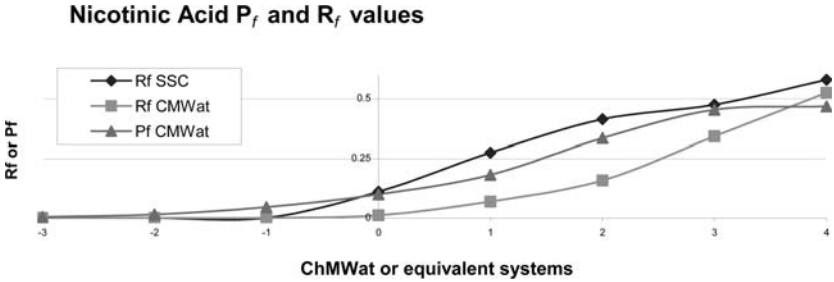

Figure 11. Comparison of nicotinic acid P_f and R_f values in ChMWat and SSC solvent systems.

compounds tend to elute at higher retention volumes than their shake-flask P values would indicate. This may to be due to additional stationary phase being displaced by the mobile phase as the run progresses.[27] It is also appropriate to observe that retention volumes are typically calculated by multiplying retention times and flow rate instead of being measured directly as volumes.[28]

There are at least 5 ways to deal with lagging compounds: (i) The mobile and stationary phases can be reversed at a convenient time during the run to elute slow-moving compounds (dual mode elution).[29] (ii) The flow rate can be increased in a stepwise manner during the run to push lagging compounds out faster with little change in solvent volumes.[30] (iii) The solvent system can be changed in a stepwise fashion to give a gradient elution effect.[31] (iv) The mobile phase may be replaced by the stationary phase at a point during the run and the coils drained with the compounds continuing to elute and be collected as fractions. (v) The elution extrusion technique recently discribed.

In the case of HEMWat systems with the aqueous phase as the mobile phase, generally, more polar compounds elute more quickly than less polar compounds. This is comparable to the "reverse" phase of solid support liquid chromatography. In this case, the relationship between the shake-flask P value, calculated by c_{UP}/c_{LP}, and $K = c_{stat}/c_{mob}$, is $K = P$, since the lower phase is mobile and the upper phase is stationary.

For a particular HEMWat number, the choice of mobile phase is an important consideration. By comparing normal phase and reverse phase it can be shown that, as expected, compounds with a shake-flask P value close to 1 will be present in the HSCCC sweet spot window in both normal and reverse phase. Therefore, for those compounds it does not matter much which phase is chosen to be mobile. However, there is a significant difference between normal phase (organic phase mobile) and reverse phase (aqueous phase mobile) as to which compounds are actually separated. As shown in Figure 12, the normal phase gathers less polar compounds (those with P values on the upper edge of the sweet spot interval), while reverse phase gathers more polar compounds with P values on the lower edge of the sweet spot interval. Therefore, the choice of mobile phase must be taken into consideration, when the HSCCC run for a particular target compound or cluster of compounds is being planned. First of all, the selection of a solvent system that hits close to $P = 1$ for the target compound(s) (the ideal HEMWat number) should be attempted. It can then be decided whether to gather higher P values (less polar behavior) by doing a normal phase run or to gather lower P values (more polar behavior) by doing a reverse phase run. The decision to go normal phase, or reverse phase may be a way of "hedging your bets" that the right solvent system was chosen or it may be based on whatever other compounds are known or believed to be present in the mixture. The choice of normal phase, or reverse phase is illustrated in Figure 12 by comparing runs of standard compounds under similar conditions

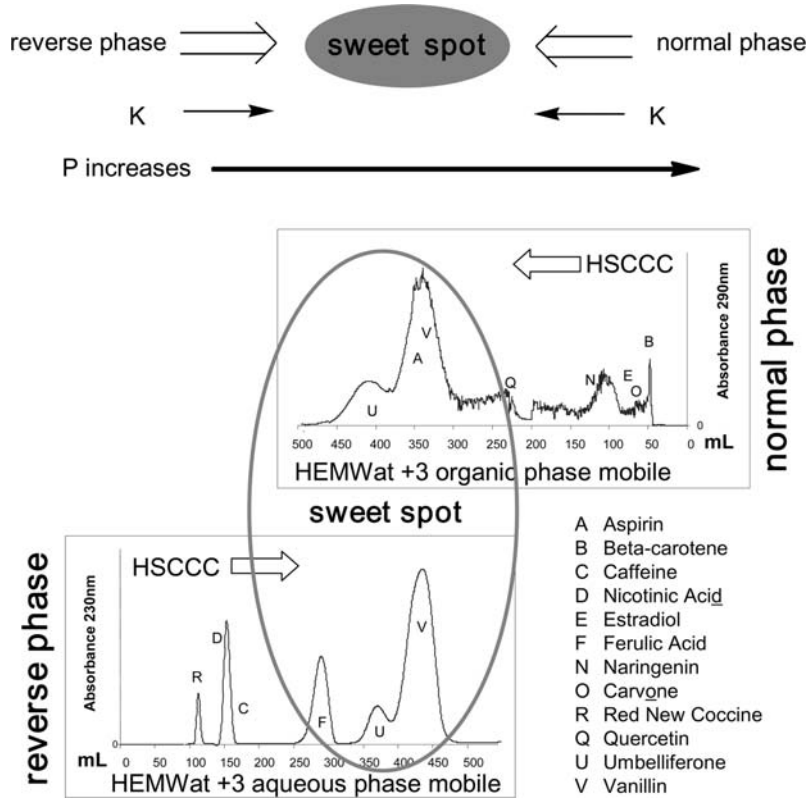

Figure 12. Schematic of the sweet spot concept, illustrating the gathering effect of normal and reverse phases in relationship to P and K (see text for definitions). Normal phase separation: HSCCC K (retention volume in mL), carotene 0 (44), carvone: 0.09 (69), estradiol 0.19 (96), naringenin 0.26 (117), quercetin 0.68 (238), vanillin 1.06 (342), aspirin 1.06 (342), umbelliferone 1.30 (409). 86% retention. Reverse phase separation: HSCCC P (retention volume in mL), new coccine red dye 0 (104), nicotinic acid 0.21 (150), caffeine 0.21 (150), ferulic acid 0.82 (286), umbelliferone 1.2 (367), vanillin 1.49 (433). 68% retention.

in both normal and reverse phase. There are three compounds that hit the sweet spot in both normal and reverse phase. The normal phase run also separates three compounds of higher P values that elute in front of the sweet spot. The reverse phase runs separates ferulic acid, which eluted too slowly in the normal phase run, as well as more polar compounds.

A similar situation is seen in the HEMWat 0 HSCCC experiment with standard compounds (see Table 10). Vanillin and aspirin are at the polar end of the shake-flask sweet spot and do not elute at reasonable times in normal phase. However, in reverse phase vanillin and aspirin elute in the HSCCC sweet spot, and umbelliferone is also gathered in.

Table 10. Normal phase (NP) and reverse phase (RP) HSCCC P values for carvone, estradiol, coumarin, naringenin, vanillin, aspirin and umbelliferone in HEMWat 0

Code	Compound	P in HSCCC NP	P in HSCCC RP
O	Carvone	4.01	
E	Estradiol	0.74	2.01
M	Coumarin	0.66	1.83
N	Naringenin	0.42	1.12
V	Vanillin		0.68
A	Aspirin		0.68
U	Umbelliferone		0.48

The following are some other practical reasons for deciding to use normal phase or reverse phase elution for HEMWat solvent systems: (i) It may be easier to evaporate solvents from organic phase fractions. (ii) Compounds may crystallize easier from aqueous phase fractions. (iii) The CCC machine may operate at lower pressures in ascending mode and flow rates can be increased. (iv) In general, higher % retention of the stationary phase in normal phase (82–92%) as compared to reverse phase (66–87%) was observed. (v) The elution volume for acidic or basic compounds may be affected by the choice of mobile phase. For example, an amine such as reserpine tends to elute in a large volume of mobile phase in normal phase runs but elutes in a smaller volume in reverse phase runs. (vi) Reverse phase mode tends to improve UV detection capabilities. Ultimately, the decision for the elution mode has to take into account all these instrumental parameters, besides practical and sample-related considerations.

A Close G.U.E.S.S.

To summarize the above, G.U.E.S.S. methodology can be outlined as follows: G.U.E.S.S. utilizes routine TLC to determine the best solvent system to use for the CCC separation. For the HEMWat method, the most simplistic approach is to use, for TLC, the hexane/ethyl acetate mixtures (SSE) defined in Table 6. The SSE TLC solvent system that gives an R_f value closest to 0.5 for the target compound corresponds to the best choice of a HEMWat solvent system. A second approach to guide HEMWat selection that works stand-alone, or can be used as additional information, is to use G.U.E.S.S. Mix standard compounds G.U.E.S.S. Mix to calibrate the TLC. Solvent systems, defined in Table 7, that are equivalent to the SSE TLC solvent systems may be used as supporting information. Once the best CCC solvent system has been selected ("GUESSed)", and the choice of mobile phase must be taken into consideration as discussed above. After performing the CCC experiment,

adjustments can be made as necessary. One advantage of the G.U.E.S.S. method is that it allows for organized adjustment of solvent systems to achieve the optimal CCC conditions for a particular separation. The G.U.E.S.S. method is also very useful in deciding how to separate compounds of a mixture that elute outside of the sweet spot, and need to be re-purified as a result.

In practical terms, the empirical G.U.E.S.S. prediction allows the determination of the ideal, or close to ideal, HEMWat and, with limitations, ChMWat solvent system. While this is most desirable, it may not be necessary to accurately predict the ideal HEMWat number in order to observe the target compound in the CCC sweet spot. The solvent systems of the HEMWat method overlap with each other as far as their ability to capture a particular compound in the sweet spot is concerned. For example, Table 11 shows normal phase HSCCC P values in italics and reverse phase HSCCC P values in bold. Even at these HEMWat number intervals it is possible to catch a compound in the sweet spot with either normal or reverse phase in at least two or three combinations of HEMWat number and mode. By adding HEMWat -4, -2, -1, $+1$, $+2$ and $+4$ systems to the information on Table 11, the retention times and separation behavior can certainly be fine-tuned.

G.U.E.S.S. for Valerenic Acid and Acetoxy Valerenic Acid

The fourth stage of this study involved testing the G.U.E.S.S. method in a real-life separation. As part of an ongoing investigation of active principles of *Valeriana officinalis*, G.U.E.S.S. was used to estimate the best HSCCC solvent system to separate valerenic acid from aqueous methanol extracts of *V. officinalis* roots. As seen in Table 12, the valerenic acid in the crude

Table 11. *Normal phase (NP)* and reverse phase (RP) behavior of coumarin, estradiol, naringenin, vanillin and umbelliferone in HSCCC with various HEMWat systems

Compound	HEMWat −5	HEMWat −3	HEMWat 0	HEMWat +3	HEMWat +5
Coumarin NP			*0.66*		
Coumarin RP	0.38	0.52	1.83		
Estradiol NP			*0.74*	*5.40*	*8.12*
Estradiol RP	0.13	0.30	2.01		
Naringenin NP			*0.42*	*3.85*	
Naringenin RP		0.12	0.68		
Vanillin NP				*0.94*	*1.90*
Vanillin RP		0.17	0.68	1.49	
Umbelliferone NP				*0.77*	*1.90*
Umbelliferone RP	0.04	0.10	0.48	1.19	

Table 12. TLC data for valerenic acid

Code	Compound	SSE1	SSE2	SSE3	Ideal HEMWat number
O	Carvone	0.55	0.8	0.91	−6
I	Ionone	0.50	0.81	0.91	−7
	Crude valerenic acid	0.38	0.66	0.81	
	Purified valerenic acid	0.28	0.54	0.77	
M	Coumarin	0.17	0.42	0.58	−1

sample travels slightly behind carvone and ionone in 3 SSE TLC solvent systems. It was decided to try a HEMWat −5 system (SSE3 equivalent) in normal phase elution with the rationale that the K value for valerenic acid value in HEMWat −5 was greater than or equal to one as is the case with ionone and carvone.

Figure 13 shows that the middle of the valerenic acid peak was at about a third of the column volume corresponding to an HSCCC K value of 0.4, which falls in the middle of the HSCCC K values for carvone (0.6) and ionone (0.3). The fatty acid that co-eluted with valerenic acid on TLC chromatograms came out almost immediately after the solvent front and was well separated from valerenic acid.

A compound related to valerenic acid, acetoxy valerenic acid, travels near vanillin, estradiol and salicylic acid in three equivalent TLC solvent systems that correspond to HEMWat −3 (see Table 13). The HSCCC separation of acetoxy valerenic acid was done in a HEMWat −3 normal phase system,

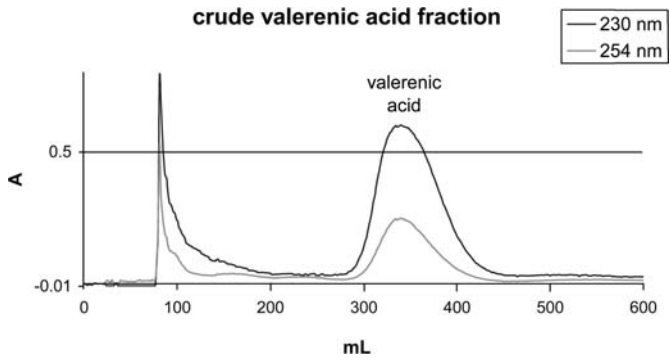

Figure 13. HSCCC UV trace of the purification of a pre-purified silica gel column fraction of valerenic acid in HEMWat –5 (7/3/7/3), 85% stationary phase retention. Organic phase mobile (T → H elution).

Table 13. TLC data from acetoxy valerenic acid in relation to G.U.E.S.S. standards with similar polarity and behavior

Code	Compound	R_f			Ideal HEMWat number
		SSE4	SSC4	SST4	
	Acetoxy valerenic acid	0.63	0.62	0.54	
Z	Salicylic acid	0.49	0.52	0.44	−3
E	Estradiol	0.52	0.48	0.44	−1
V	Vanillin	0.58	0.74	0.56	+2

with the rationale that the K value for valerenic acid value in HEMWat −3 was less than or equal to one. The middle of the acetoxy valerenic acid peak was at about a half-column volume representing a K value of 0.6. A good separation was realized between acetoxy valerenic acid and two unknown compounds that had almost identical TLC R_f values (Fig. 14).

CONCLUSIONS

Because of the large variability of natural products, the choice of solvent system in CCC separations is particularly crucial. The G.U.E.S.S. method allows a reasonable first choice based on routine TLC parameters *without* the need for additional experiments. Since TLC is routinely performed to screen extracts and monitor fractionations, the G.U.E.S.S. information essentially comes free, and simultaneously allows the determination of a CCC

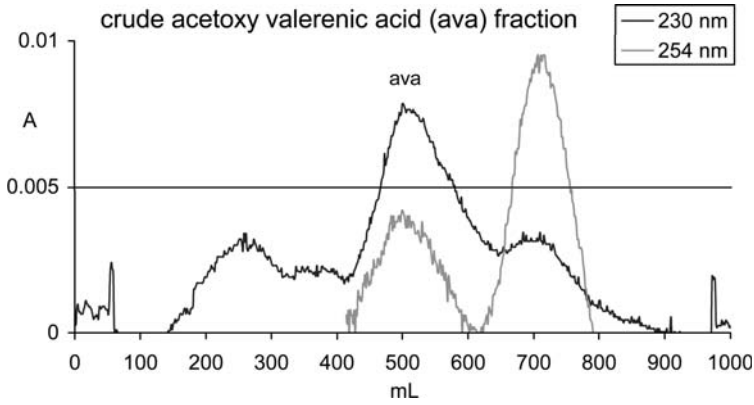

Figure 14. HSCCC trace of the purification of pre-purified HSCCC fractions of acetoxy valerenic acid (ava) in HEMWat −3 (6/4/6/4), 94% stationary phase retention.

solvent system in close proximity to the sweet spot. Consequently, the initial CCC run already yields acceptable results, provided that care is taken with regards to the choice of normal *vs.* reverse phase.

The HEMWat G.U.E.S.S. method, as summarized in Figure 2, characterizes a useful and versatile method that offers not only a set of similar solvent systems, but also a rational method for solvent system selection. TLC R_f values will often give "good enough" predictions even with simple single-phase mixtures. The same and/or additional information can be acquired from TLC by calibrating TLC with a mixture of G.U.E.S.S. standard compounds (G.U.E.S.S. Mix, Figure 3) and by the employment of equivalent solvent systems (Table 7). The usefulness of the ChMWat G.U.E.S.S. method appears to be more related to chemical properties of the analytes (acidity, bascity, etc.) and, therefore, requires further development targeted towards certain compound classes. In the meantime, there is an alternative way of selecting optimal chloroform/methanol/water solvent systems reported previously that may be helpful.[20]

Because the G.U.E.S.S. system provides more rapid, routine access to tuned CCC separation power, its applicability covers the whole breadth of natural products research. The obvious examples are the targeted purification of reference compounds, and the bioassay-guided fractionation of natural extracts. But also in the field of metabolome analysis, G.U.E.S.S.-based CCC will facilitate the systematic analysis of all metabolites from complex matrices, by allowing sorting them based on P (or K) values. Now that the proof of principle for routine "G.U.E.S.S.work" has been demonstrated, it is a logical extension to include less polar (substitution of methanol) and more polar (addition butanol) modifications of HEMWat and ChMWat in future studies.

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